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Quantitative determination of oseltamivir and oseltamivir carboxylate in human fluoride EDTA plasma including the *ex vivo* stability using high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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ABSTRACT

Oseltamivir, the ethyl ester prodrug of the neuramidase inhibitor oseltamivir carboxylate, is licensed for the treatment of patients with influenza virus infection. Here we describe the development and validation of an assay for the simultaneous quantification of oseltamivir and oseltamivir carboxylate in human fluoride EDTA plasma including the *ex vivo* stability using liquid chromatography coupled to tandem mass spectrometry. Sample pretreatment consisted of protein precipitation with 8% (v/v) trichloroacetic acid in water using only 50 μL plasma. Chromatographic separation was performed on a reversed phase C18 column (150 mm × 2.0 mm ID, particle size 4 μm) with a stepwise gradient using 0.1% formic acid and methanol at a flow rate of 250 μL/min. A triple quadrupole mass spectrometer operating in the positive ionization mode was used for detection and drug quantification. The method was validated over a range of 3–300 ng/mL for oseltamivir and 10–10,000 ng/mL for oseltamivir carboxylate. Deuterated oseltamivir and oseltamivir carboxylate were used as internal standards. The intra-assay accuracies and precisions for oseltamivir were between −8.8 and 16.3% at the LLOQ level, whereas for all other concentration levels this was −8.6 and 14.5%. For oseltamivir carboxylate the intra-assay accuracies and precisions were between −10.9 and 10.7% at all levels. Furthermore, oseltamivir was stable in plasma and whole blood *ex vivo* in commercially available fluoride EDTA tubes for at least 24 h at 2–8 °C. This method is now applied for the determination of both compounds in specific patient populations to evaluate current dosing guidelines.

1. Introduction

Oseltamivir (Tamiflu®, Hoffmann-La Roche, Nutley, NJ; Fig. 1A) is an ethyl ester prodrug which is rapidly hydrolyzed *in vivo* by liver carboxylesterases to the active metabolite oseltamivir carboxylate (Fig. 1B) [1]. Oseltamivir carboxylate is a neuramidase inhibitor with potent activity against the 2009–2010 H1N1 influenza pandemic [2]. During the recent pandemic, oseltamivir was often prescribed in patients at risk for complicated illness due to influenza, like children, elderly and immunocompromised patients. However, relatively few studies have been published on the pharmacokinetics of oseltamivir and oseltamivir carboxylate in these specific patient populations [3–7]. Dosing regimens are thus based on limited information, which raised questions whether these fragile populations were adequately treated within current dosing guidelines [4]. Determination of oseltamivir and oseltamivir carboxylate levels may help to evaluate current dosing guidelines.

Previously, several assays for the determination of oseltamivir and oseltamivir carboxylate in various biological fluids have been described [8–13]. These methods all use solid phase extraction (either on-line or off-line) as sample pretreatment, which can be time consuming and expensive. To the best of our knowledge, no validated method has been published for the determination of oseltamivir and oseltamivir carboxylate in plasma using a simple protein precipitation as sample pretreatment.

The *ex vivo* stability of oseltamivir is a major hurdle for performing pharmacokinetic studies in a hospital setting of critically ill patients. Therefore, the stability of both analytes in several collection tubes has been determined, since human plasma esterases may cause variable and extensive *ex vivo* conversion of oseltamivir into oseltamivir carboxylate [14,15].

This paper describes the development of a bioanalytical assay to determine oseltamivir and oseltamivir carboxylate plasma concentrations using high performance liquid chromatography coupled to

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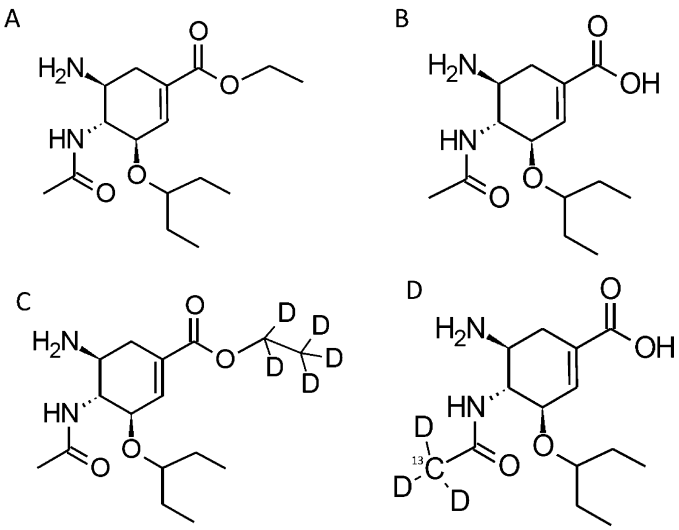

Fig. 1. Chemical structures of (A) oseltamivir, (B) oseltamivir carboxylate, (C) [$^2H_5$]-oseltamivir and (D) [^{13}C,$^2H_2$]-oseltamivir carboxylate.

tandem mass spectrometry (HPLC–MS/MS). Additionally, the stability of both analytes in plasma and whole blood in commercially available fluoride EDTA tubes was assessed, to allow simple sample handling in clinical settings.

2. Experimental

2.1. Chemicals and reagents

Oseltamivir originated from Hoffmann-La Roche Ltd. (Basel, Switzerland) while oseltamivir carboxylate was obtained from Toronto Research Chemicals (North York, ON, Canada). The internal standards [$^2H_5$]-oseltamivir and [^{13}C,$^2H_3$]-oseltamivir carboxylate (Fig. 1C and D, respectively) were purchased from Alsachim (Illkirch Graffenstaden, France). Methanol was obtained from Biosolve Ltd. (Valkenswaard, The Netherlands). Distilled water originated from B. Braun (Melsungen, Germany). Formic acid was from Merck (Amsterdam, The Netherlands). Drug free EDTA and fluoride EDTA plasma and whole blood were obtained from healthy volunteers using 4 mL dipotassium EDTA tubes and sodiumfluoride/disodium EDTA (6.0 mg) tubes from BD Vacutainer (Plymouth, United Kingdom), respectively.

2.2. Chromatographic conditions

Chromatographic separation of oseltamivir and oseltamivir carboxylate was carried out using a Shimadzu LC system (Shimadzu, Kyoto, Japan) consisting of a SIL-HTc autosampler with SCL-10A^{vp} systemcontroller, two LC20-AD Prominence pumps, a FCV-11AL valve unit, a DGU-20A5 inline degasser unit and a CTO-20AC column oven. Compounds were eluted using a stepwise gradient (Table 1) at a flow rate of 250 μL/min. Mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B consisted of 100% methanol. A Synergi Hydro C18 column (150 mm × 2.0 mm ID, 4 μm particle size; Bester, Rotterdam, The Netherlands) protected with a pre-column (Gemini C18 pre-column, 4.0 mm × 2.0 mm ID; Phenomenex, Torrance, CA, USA) was used for separation. The column outlet was connected to an electrospray ionization (ESI) sample inlet through a divert valve. The divert valve was directed to waste during the first and last 2 min of the run to prevent the introduction of endogenous compounds into the mass spectrometer. Total run time was 9 min and sample injections of 10 μL were carried out with the autosampler thermostated at 4 °C.

Table 1
Stepwise gradient for the HPLC–MS/MS experiments.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
0.5	80	20
2.0	20	80
7.0	20	80
7.1	80	20
9.0	80	20

2.3. Mass spectrometric conditions

A Thermo Fischer TSQ Quantum Ultra^{TM} mass spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, USA) with an ESI source operating in the positive ion mode was used. The sheath and auxiliary gasses were set at 40 and 2 arbitrary unit (au), respectively, with the ion sweep gas set at 2.0 au (all N_2). The spray voltage was 3.5 kV. The capillary was heated to 350 °C. The collision energy was 20 au for all compounds. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired. The precursor/product ion transitions were *m/z* 313/166 for oseltamivir, *m/z* 285/138 for oseltamivir carboxylate, *m/z* 318/171 for [$^2H_5$]-oseltamivir and *m/z* 289/138 for [^{13}C,$^2H_3$]-oseltamivir carboxylate. Data were acquired using Xcalibur 2.0 and processed using LCQuan 2.5 software (Thermo Fisher Scientific Inc.).

2.4. Preparation of calibration standards and quality control samples

Stock solutions of oseltamivir and its active metabolite oseltamivir carboxylate were prepared from independent weighings; one for calibration standards (CAL) and one for validation samples (VS). Approximately 1 mg of both compounds was accurately weighted (compound weighing was corrected for potency) and dissolved in 1 mL of water using calibrated pipettes to give 1 mg/mL stock solutions. The stock solutions of the internal standards were also prepared in water with an approximate concentration of 1 mg/mL.

For the preparation of the calibration standards, working solutions in the range from 120 to 12,000 ng/mL and 400 to 400,000 ng/mL were used for oseltamivir and oseltamivir carboxylate, respectively. The working solutions were prepared by diluting the oseltamivir and oseltamivir carboxylate stock solutions in water. To obtain calibration standards, 25 μL of each working solutions was added to 950 μL of plasma to obtain calibration standards in the range from 3 to 300 ng/ml for oseltamivir and 10 to 10,000 ng/mL for oseltamivir carboxylate.

For the preparation of the validation samples working solutions in the range of 120–10,800 ng/mL and 400–360,000 ng/mL for respectively oseltamivir and oseltamivir carboxylate were prepared by dilution of independently prepared oseltamivir and oseltamivir carboxylate stock solutions. To obtain validation samples of 3, 9, 30 and 270 ng/mL for oseltamivir and 10, 30, 500 and 9000 ng/mL for oseltamivir carboxylate 25 μL of each working solution was added to 950 μL of plasma. The stock and working solutions were stored at nominally −20 °C until use.

2.5. Sample pretreatment

To precipitate plasma proteins 100 μL 8% (v/v) trichloroacetic acid (TCA) in water was added to 50 μL plasma. Before precipitation, 20 μL internal standard working solution with 62.5 ng/mL [$^2H_5$] oseltamivir and 1250 ng/mL [^{13}C,$^2H_3$] oseltamivir carboxylate in water was added. After vortex mixing for 10 s, samples were

centrifuged at 10,000 × *g* for 10 min. Finally, 100 μL of clear supernatant was transferred to an autosampler vial and 10 μL of the clear supernatant was injected onto the column. The internal standard working solutions were stored at nominally −20 °C until use, whereas 8% TCA was stored at room temperature.

2.6. Validation

For the validation of the assay the linearity, accuracy, precision, specificity, selectivity, recovery, ion suppression, carry over and stability under several conditions were determined according to the FDA guidelines for validation of bioanalytical assays [16].

2.7. Application of the assay

A full pharmacokinetic curve at steady state was drawn from a patient admitted to an intensive care unit with severe complications from a H1N1 infection receiving 75 mg oseltamivir twice daily. At each time point venous blood was collected in 4 mL fluoride EDTA tubes.

3. Results

3.1. Assay development

During the optimization of the mass spectrometric parameters, the Q1 spectra of oseltamivir and oseltamivir carboxylate showed singly charged molecular ions at *m/z* 313 and 285, respectively. For the internal standards, [$^2H_5$] oseltamivir and [^{13}C,$^2H_3$]-oseltamivir carboxylate, the most intense ions also corresponded to the singly charged molecular ions at *m/z* 318 and 289, respectively. MS/MS experiments were carried out to determine the most abundant product ions for MRM. The most intense product ion fragments for oseltamivir, oseltamivir carboxylate, [$^2H_5$] oseltamivir and [^{13}C,$^2H_3$] oseltamivir carboxylate were observed at *m/z* 166, 138, 171 and 138, respectively. The product ion spectra with the proposed fragmentation pathways for oseltamivir and oseltamivir carboxylate are shown in Fig. 2. The retention times of oseltamivir and oseltamivir carboxylate were 5.0 and 4.7 min, respectively, under the prescribed chromatographic conditions. Typical chromatograms are depicted in Fig. 3. At the LLOQ level (3 ng/mL for oseltamivir and 10 ng/mL for oseltamivir carboxylate) a signal to noise ratio (S/N-ratio) between 5 and 10 was obtained.

As mentioned before, thus far only solid phase extraction procedures have been described as sample pretreatment [8–13]. However, this procedure can be time consuming. Therefore, theoretical assessment of the optimal protein precipitation agent for sample clean-up was performed. Zinc sulphate, acetonitrile and trichloroacetic acid were found to be the most efficient and reproducible agents to precipitate proteins with a precipitant to plasma ratio of 2:1 [17]. Zinc sulphate can cause significant source contamination and was thus excluded for the precipitation of plasma proteins. Since oseltamivir and oseltamivir carboxylate are highly soluble in water, no compound loss was seen when using trichloroacetic acid. The extraction recovery was high and reproducible at all concentration levels (Table 2). Therefore, trichloroacetic acid was chosen as protein precipitation agent.

3.2. Validation

3.2.1. Linearity

Eight non-zero calibration samples were prepared and analyzed in duplicate in three separate runs. The linear regression of the ratio of the areas of the analyte and the internal standard peaks *versus* the concentration were weighted by using $1/x$ and $1/x^2$ as weighting factors. Back-calculated concentrations were determined in order to establish the best weighting factor. The model with the lowest total bias and the optimal consistency of bias across the concentration range was used for further quantification. Linearity was evaluated by means of back-calculated concentrations of the calibration standard. The assay was linear over the validated range from 3 to 300 ng/mL for oseltamivir and 10 to 10,000 ng/mL for oseltamivir carboxylate with a weighting factor of $1/x$ and $1/x^2$, respectively. All correlation coefficients (r^2) were at least 0.9935. At all levels the accuracies were within ±15% with CV values less than 10.1%, which is in accordance with the FDA guidelines.

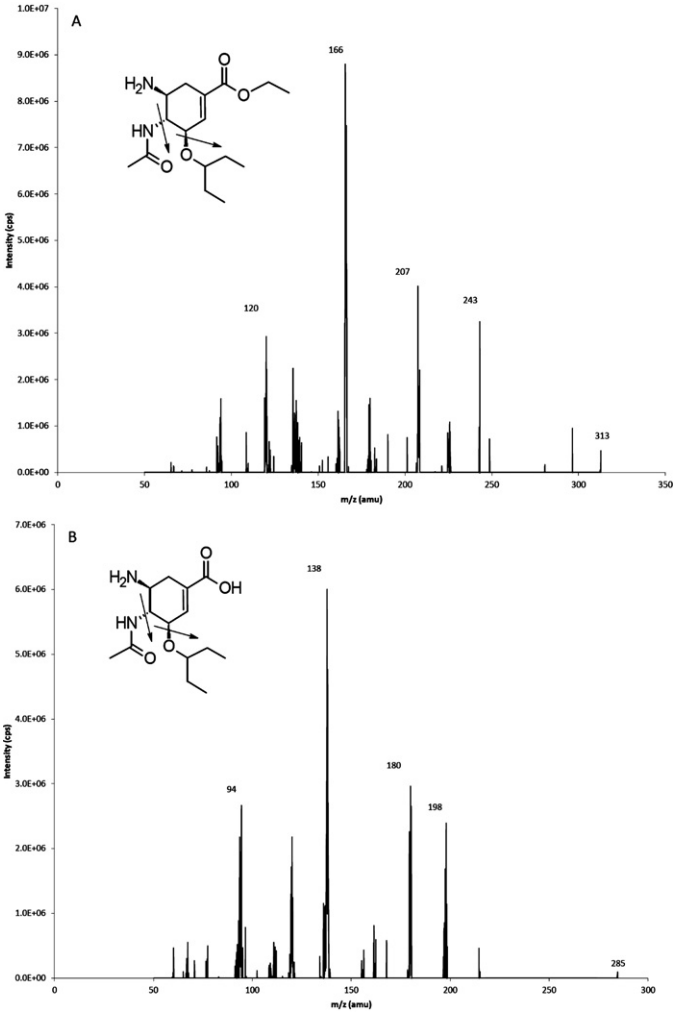

Fig. 2. Positive MS/MS production spectra for (A) oseltamivir (precursorion *m/z* 313) and (B) oseltamivir carboxylate (precursorion *m/z* 285) with proposed fragmentation pathways.

3.2.2. Accuracy and precision

Accuracy and precision were determined by quantification of validation samples with analyte concentrations at the LLOQ and in the low, mid and high ranges of the calibration curves. Each validation sample was analyzed in 5 replicates in 5 separate analytical runs. The accuracy was defined as the percentage difference between the calculated concentration and the nominal concentration. The intra- and inter-assay accuracies should be within ±20% for the lower limit of quantification (LLOQ) and ±15% for the low, mid and high concentrations. The precision was reported using the coefficient of variation (CV%). The precisions CV% should be less than 20% for the LLOQ and less than 15% for the low, mid and high concentrations. In Table 2 the inter-assay accuracies and the within run and between run precision is summarized for the

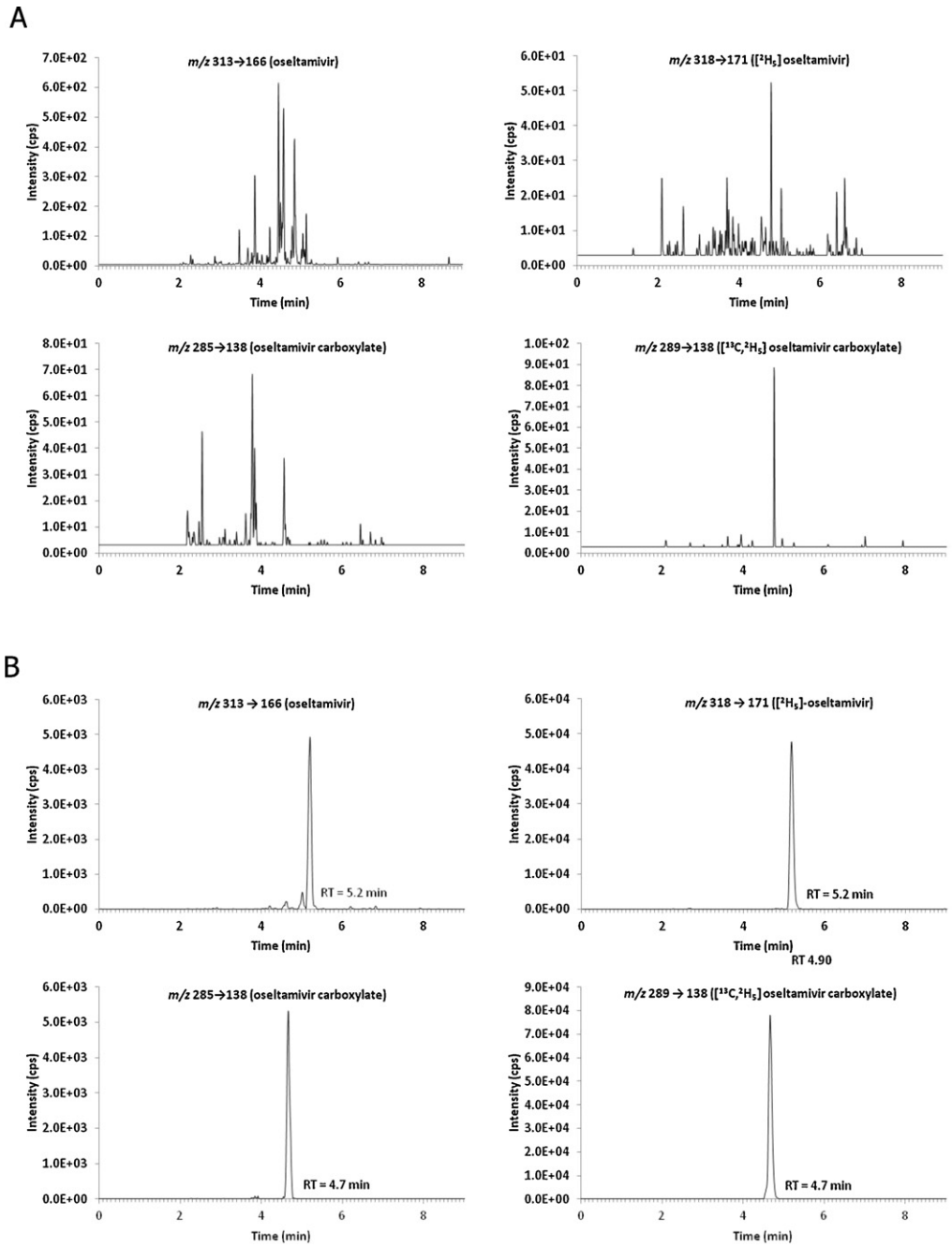

Fig. 3. MRM chromatograms of control human fluoride EDTA plasma (A) and a plasma sample spiked at the LLOQ level (B, 3 ng/mL oseltamivir carboxylate). RT; retention time.

Table 2
Assay performance for oseltamivir and oseltamivir carboxylate.

Analyte	Nominal concentration (ng/mL)	Inter-assay accuracy (% DEV)	Within run precision (% CV)	Between run precision (% CV)	No. of replicates
Oseltamivir	2.99	0.38	16.3	8.34	25
	8.96	−3.96	8.16	2.52	25
	29.9	−5.07	5.04	1.91	24
	269	2.34	14.5	2.07	25
Oseltamivir carboxylate	9.79	−1.08	10.4	8.06	25
	26.4	6.55	5.02	1.48	23
	489	−0.85	5.44	4.40	24
	8807	3.37	7.71	1.42	25

DEV, deviation; CV, coefficient of variation.

Table 3
Extraction recoveries for oseltamivir and oseltamivir carboxylate for human fluoride EDTA plasma.

Analyte	Nominal conc. (ng/mL)	Recovery (%)	RSD (%)	No. of replicates
Oseltamivir	8.96	103	3.47	3
	29.9	101	5.01	3
	269	88.6	4.78	3
Oseltamivir carboxylate	26.4	106	5.35	3
	489	104	2.51	3
	8807	103	7.46	3

Conc., concentration; RSD, relative standard deviation.

tested concentration levels. The intra-assay accuracies and precisions for oseltamivir were between −8.8 and 16.3% at the LLOQ level, whereas for all other concentration levels this was −8.6 and 14.5%. For oseltamivir carboxylate the intra-assay accuracies and precisions were between −10.9 and 10.7% at all levels. Therefore, the accuracy and precision of the method comply to the FDA criteria for bioanalytical method validation.

3.2.3. Specificity and selectivity

Six different batches of control drug-free fluoride EDTA plasma were prepared as double blanks and spiked at the LLOQ level to determine whether endogenous compounds from plasma interfered with the detection of the analytes or internal standard. No interferences of endogenous compounds (≤0.5% of LLOQ levels) were observed at the retention time of oseltamivir. At the retention time of oseltamivir carboxylate the interference was ≤12.3% of LLOQ levels. According to the guideline the areas of peaks co-eluting with the analytes should not exceed 20% of the area at the LLOQ level, whereas the deviation of the nominal concentration for the LLOQ samples should be within ±20%. Therefore, the specificity and selectivity was found to be acceptable.

3.2.4. Recovery and ion suppression

The protein precipitation recovery of oseltamivir and oseltamivir carboxylate were determined at three concentrations by comparing the analytical results of the processed samples with those of processed blank samples spiked with analyte. Table 3 shows the recovery was high (88.6–106%) and reproducible (RSD ≤ 7.46%) at all concentration levels.

The matrix effect was examined by comparing the analytical response of processed blanks spiked with analyte with those unprocessed samples at medium concentration level in precipitation reagent. These experiments were performed in triplicate. The mean matrix factor detected for oseltamivir in plasma was 1.74 (range 1.45–1.95), whereas for oseltamivir carboxylate in plasma this was 0.852 (range 0.818–0.869). Thus, an ion enhancement was seen for oseltamivir, whereas the matrix effect was minimal for oseltamivir carboxylate. Additionally, the internal standard-normalized matrix factor was calculated at low and high level in triplicate using the peak response ratio (analyte/internal standard). For oseltamivir at low and high concentration this was 0.924 (CV = 6.1%) and 0.941 (CV = 4.1%), whereas for oseltamivir carboxylate this was 1.04 (CV = 3.8%) and 1.02 (CV = 1.2%) at low and high concentration, respectively. Thus, the internal standards corrected for the ion enhancement and suppression, since the internal standard-normalized matrix factor is close to 1. The CV for the internal standard-normalized matrix factor was ≤15% for both analytes, which is within common acceptance criteria [18].

3.2.5. Carry over

Carry-over was tested by injecting two processed blank matrix samples sequentially after injecting an upper limit of quantification (ULOQ) sample. The carry over for oseltamivir and oseltamivir carboxylate was ≤0.3 and 15.9% of the response detected in a LLOQ sample, respectively. No carry over was found for both internal standards. Since the response in the first blank matrix at the retention time of oseltamivir, oseltamivir carboxylate and both internal standards should be less than 20% of the response detected in a LLOQ sample the carry over of the analytes and internal standards was considered acceptable.

3.2.6. Stability

The stability of oseltamivir and oseltamivir carboxylate was investigated during various steps of the analysis. The oseltamivir and oseltamivir carboxylate stock solution stability was investigated after storage at −20 °C for 2 years and 1 month, respectively. The stock solution was considered stable when 95–105% of the nominal concentration was found compared to freshly prepared stock solution. Both stocks fulfilled these criteria since at least 95% of the nominal concentration was found when compared to a fresh solution after the indicated storage times (Table 4).

The stability of oseltamivir and oseltamivir carboxylate in fluoride EDTA plasma was investigated in triplicate at low and high concentration levels after three freeze–thaw cycles (Table 4). Additionally, the *ex vivo* stability of oseltamivir was investigated. Previously, oseltamivir was proven stable in fluoride-oxalate tubes [14]. Since oxalate and fluoride are both inhibitors of carboxylesterase, the stability of oseltamivir in commercially available fluoride EDTA tubes was studied. Oseltamivir (289 ng/mL) was spiked to fresh EDTA and fluoride EDTA plasma from 6 healthy volunteers. Oseltamivir and oseltamivir carboxylate concentrations were determined in duplicate after $t = 0, 0.5, 1, 4, 8$ and 24 h incubation at 37 °C and 2–8 °C. Additionally, stability of oseltamivir was assessed in EDTA and fluoride EDTA whole blood from 1 healthy volunteer in triplicate at $t = 0, 1, 2, 4, 8$ and 24 h after incubation at 37 °C and 2–8 °C. For these experiment, whole blood spiked with oseltamivir (289 ng/mL) was incubated for 30 min at 37 °C to allow equilibration. After equilibration, whole blood was transferred to fluoride EDTA and EDTA tubes. Whole blood samples were centrifuged 5 min at 3000 rpm to obtain plasma. Oseltamivir was considered stable when the concentrations at all time points were within 85–115% of the concentration at $t = 0$ and the oseltamivir carboxylate concentration was below the LLOQ. Table 4 shows the *ex vivo* stability of oseltamivir in potassium and fluoride EDTA plasma after 24 h of storage at 37 °C and 2–8 °C. The results over time (and thus the *ex vivo* formation of oseltamivir carboxylate) are presented in Fig. 4. As expected, oseltamivir was not stable in EDTA plasma after 24 h at both 2–8 °C and 37 °C, since oseltamivir degradation was >15% and the measured oseltamivir carboxylate levels were >LLOQ of 10 ng/mL. Instability was also demonstrated for oseltamivir fluoride EDTA plasma at 37 °C after 24 h, since the oseltamivir carboxylate concentration was >LLOQ. Stability was acceptable up to 8 h. At 2–8 °C oseltamivir was found stable in fluoride EDTA plasma up to 24 h since the oseltamivir carboxylate was <LLOQ and the measured oseltamivir concentration within 7.61% (±15%) of the initial concentration.

For oseltamivir in whole blood comparable results were obtained; after 24 h at 2–8 °C and up to 8 h at 37 °C oseltamivir in fluoride EDTA was found stable (deviation of initial concentration was −13.8% and 7.43%, respectively). The amount of oseltamivir

Table 4
Stability of oseltamivir and oseltamivir carbxylate in working solution and biomatrix.

Matrix	Condition	Analyte	Initial conc. (ng/mL)	Meas conc. (ng/mL)	DEV (%)	CV (%)	No. of replicates
Water (stock)	2 years, −20 °C	Oseltamivir	1.000.000	984.300	−1.57	1.61	3
	1 month, −20 °C	Oseltamivir carboxylate	1.000.000	954.500	−4.55	1.59	3
Fluoride EDTA plasma	3 freeze (−20 °C)/thaw cycles	Oseltamivir	8.96	7.65	−14.6	0.66	3
			269	244	−9.29	4.64	3
		Oseltamivir carboxylate	26.4	29.3	11.0	4.91	3
			8807	9472	7.55	1.36	3
	24 h, 37 °C	Oseltamivir	289	250	−13.6	8.37	6
		Oseltamivir carboxylate	–	13.8	NA	NA	6
	24 h, 2–8 °C	Oseltamivir	289	311	7.61	2.81	6
		Oseltamivir carboxylate	––	<LLOQ	NA	NA	6
EDTA plasma	24 h, 37 °C	Oseltamivir	289	96.1	−66.8	4.23	6
		Oseltamivir carboxylate	–	156	NA	NA	6
	24 h, 2–8 °C	Oseltamivir	289	243	−15.9	6.61	6
		Oseltamivir carboxylate	–	57.8	NA	NA	6
Final extract	4 days, 2–8 °C	Oseltamivir	8.96	9.12	1.79	1.48	3
			269	292	8.55	1.42	3
		Oseltamivir carboxylate	26.4	28.2	6.82	3.01	3
			8807	9066	2.94	1.49	3
	3 days, reinjection, autosampler (4 °C)	Oseltamivir	8.96	8.84	−1.34	1.31	3
			269	280	4.09	4.84	3
		Oseltamivir carboxylate	26.4	29.3	11.0	4.00	3
			8807	9741	10.6	0.73	3

Conc., concentration; Meas., measured; DEV, deviation; CV, coefficient of variation; NA, not applicable; No., number.

carboxylate found was <LLOQ at both time points. These results demonstrate that when patients samples are collected in fluoride EDTA tubes, sample treatment times are not critical.

The reinjection reproducibility and the final extract stability was determined after storage at 2–8 °C at three concentrations in triplicate. Reinjection was found reproducible since 102–109% of the nominal concentration was recovered. The final extracts were also stable; 99–111% of the nominal concentrations was recovered when compared with freshly prepared calibration standards.

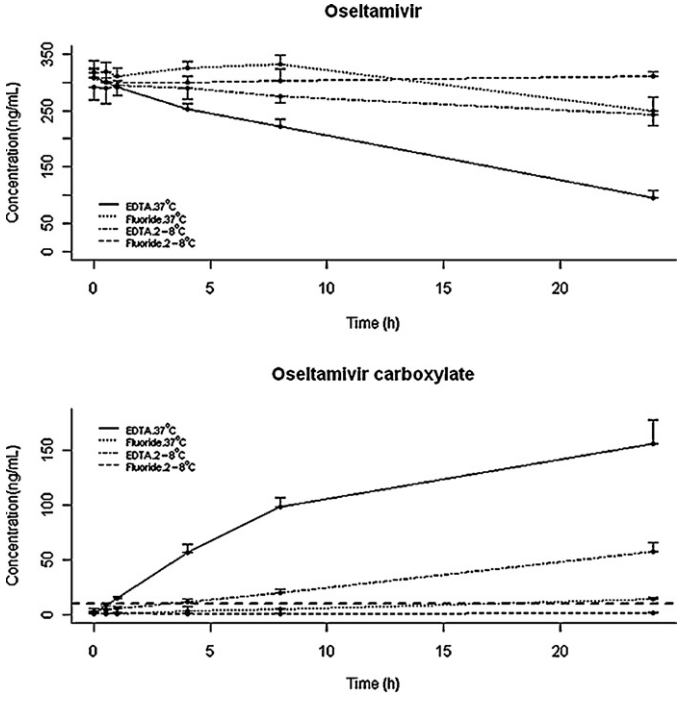

Fig. 4. Concentration of oseltamivir and oseltamivir carboxylate in potassium EDTA and fluoride EDTA plasma during 24 h at 37 °C and 2–8 °C. The error bars represent the standard deviation (SD).

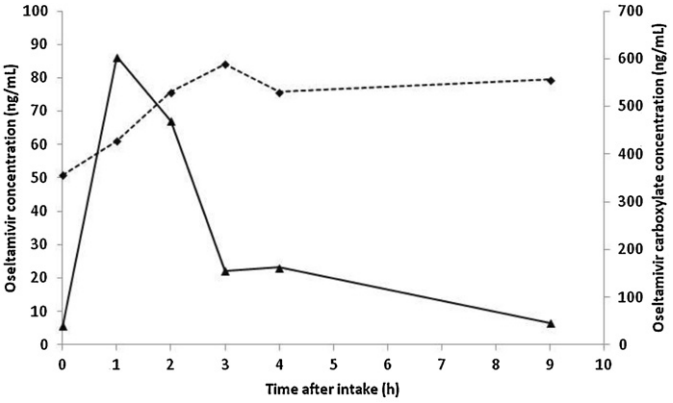

Fig. 5. Full pharmacokinetic curve at steady of oseltamivir and oseltamivir carboxylate in a patient on a twice daily 75 mg oseltamivir dose. The solid line is the oseltamivir concentration and the dashed line the oseltmivir carboxylate concentration.

3.3. Application of the assay

The full pharmacokinetic curve of oseltamivir and oseltamivir carboxylate of a patient treated with oseltamivir on an intensive care unit is depicted in Fig. 5. The oseltamivir carboxylate concentrations are similar to previously reported concentrations in critically ill patients, with a maximum oseltamivir carboxylate concentration of 589 ng/mL 3 h after intake [19].

4. Discussion

The described bioanalytical method provides an efficient tool for monitoring oseltamivir and oseltamivir carboylate concentrations in critically ill patients hospitalized due to influenza in case questions exist whether these patients are adequately treated within current dosing strategies.

Previously, several articles have been published on the determination of oseltamivir and oseltamivir carboxylate in human plasma [8,10–12]. Although total run times of the previously described methods were shorter than the currently described method (7, 4, 3.6 and 1 min *versus* 9 min), the sample pretreatment was more

complex. Previous methods used solid phase extraction as sample pretreatment. This can be a time consuming and expensive process since trained personnel and specialized equipment is required. Heinig and Bucheli dealt with this problem by using an online solid phase extraction system, which enables high throughput analysis [11]. However, most hospital laboratories do not have these facilities. Therefore, a simple sample pretreatment combined with a straightforward HPLC–MS/MS method is ideal for a hospital setting where monitoring oseltamivir and oseltamivir carboxylate concentrations is required. The LLOQ of such assay is not necessarily as low as possible, but covers the expected therapeutical range. The minimum plasma concentration (C_{min}) of oseltamivir carboylate reported in healthy adults receiving 75 mg twice daily oseltamivir is 168 ng/mL (CV 19%). Although others have reported a LLOQ of 5.22 ng/mL and 1 ng/mL, the described assay also has an acceptable LLOQ since 10 ng/mL lies far below the C_{min} [8,11,20].

5. Conclusion

A bioanalytical method for the determination of oseltamivir and its active metabolite, oseltamivir carboxylate has been developed. The method is simple, specific and reproducible and can be used for monitoring oseltamivir and oseltamivir carboxylate in patients with influenza infection. When whole blood is collected in fluoride EDTA tubes, oseltamivir stability has been demonstrated for 8 h at 37 °C and 24 h at 2–8 °C. In fluoride EDTA plasma, the analytes were stable for at least 24 h at 2–8 °C. Using fluoride EDTA tubes, sample pretreatment under refrigerated conditions is not critical.

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